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Journal of Chromatography B, 778 (2002) 99–111

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

A multi-analyte method for the quantification of contemporary pesticides in human serum and plasma using high-resolution mass spectrometry[q]

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Abstract

We have developed a sensitive and accurate analytical method for quantifying 29 contemporary pesticides in human serum or plasma. These pesticides include organophosphates, carbamates, chloroacetanilides, and synthetic pyrethroids among others and include pesticides used in agricultural and residential settings. Our method employs a simple solid-phase extraction followed by a highly selective analysis using isotope dilution gas chromatography–high-resolution mass spectrometry. Our method is very accurate, has limits of detection in the low pg/g range and coefficients of variation of typically less than 20% at the low pg/g end of the method linear range. We have used this method to measure plasma pesticide concentrations in females living in an urban area. We found detectable concentrations of carbaryl/naphthalene, propoxur, bendiocarb, chlorpyrifos, diazinon, dicloran, captan and folpet or their metabolites in more than 20% of the plasma samples tested.
Published by Elsevier Science B.V.

Keywords: Pesticides

1. Introduction

In 1997, about five billion pounds of pesticide active ingredients were applied in the United States with about 75% of the applications for agricultural use [1]. The most recent registration data provided by the US Environmental Protection Agency show over 800 pesticidal active ingredients available in about 21 000 different formulations [1]. The widespread use of the so-called contemporary or current-use pesticides makes it virtually impossible for the average person to totally avoid exposure. Because it is inevitable that humans will be exposed to a variety of toxicants including contemporary pesticides during a lifetime, the risks associated with these exposures must be appropriately evaluated.

[q]The use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service, the Department of Health and Human Services, or the Centers for Disease Control and Prevention.

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1570-0232/02/$ – see front matter Published by Elsevier Science B.V.
PII: S0378-4347(01)00444-3

ED_001449_00000295

Exposure assessment is an integral component of risk assessment. But often, reliable exposure assessment information is lacking in quantity or quality. Because human exposure to pesticides is multi-media and multi-route and varies with the use of pesticides, environmental monitoring of exposure must account for the concentration of the pesticide in all media, the time in contact with each medium, and route(s) of exposure in order to accurately calculate aggregate exposure information to a given pesticide. But even when all of this information is considered, measurements of the external dose may not accurately reflect the absorbed dose, known as the internal dose.

Because of their inherent chemical nature, contemporary pesticides have biological half-lives on the order of hours to a few days, much shorter than other organic toxicants like PCBs and dioxins, which have half-lives spanning years [2]. Therefore, the contemporary pesticides do not circulate in the bloodstream for extended periods of time, nor do they generally accumulate in tissues to any appreciable degree. These pesticides are usually metabolized rapidly, and the more polar metabolites are excreted in the urine.

Because the metabolites of pesticides are usually excreted in urine soon after exposure and because urine is usually a plentiful matrix and easy to obtain, biological monitoring of exposure to contemporary pesticides has typically involved quantifying pesticide metabolites in urine [2]. In addition, concentrations of pesticides and/or their metabolites in urine are typically much higher than in blood and are detectable for a longer period of time. However, this approach is not without its limitations. Often the human metabolites of pesticides are not known and calibration materials may not be commercially available. Additionally, urine is not a regulated matrix and 'spot' or 'grab' samples must be corrected for urine dilution to allow interindividual comparisons. For most contemporary pesticides, the best method for correcting for urine dilution is currently under debate, especially where comparisons among adults and children are desired.

Measuring the internal dose of toxicants in blood has several advantages over measuring it in urine. Generally, the parent compound, instead of a metabolite, can be directly monitored in blood products such as whole blood, plasma, or serum; therefore, the development of a blood measurement technique usually does not require detailed information on the metabolism. Also, the measurement of the intact pesticide in blood instead of a metabolite in urine yields much more accurate information as to which pesticide one was exposed. For example, measurement of 3,5,6-trichloropyridinol (3,5,6-TCPy) in urine indicates exposure to chlorpyrifos and/or chlorpyrifos-methyl. We cannot distinguish between these two pesticides based upon the metabolite information. However, if we measure these two pesticides in blood, we can qualitatively differentiate them from one another. Furthermore, measurement of the urinary metabolite 3,5,6-TCPy may indicate exposure to the pesticides themselves or their environmental degradation product which is identical to the metabolite. Distinguishing between exposure to each pesticide and exposure to their respective degradation products is very important in risk assessment because the toxicities, and hence the acceptable daily intake for chlorpyrifos, chlorpyrifos-methyl, and 3,5,6-TCPy all differ.

Because blood is a regulated fluid(i.e., the volume does not vary substantially with water intake or other factors), the blood concentrations of toxicants measured at a specified time interval after exposure will be the same as long as the absorbed amounts are constant; thus, no corrections for dilution are necessary. Blood concentrations of the toxicant are often at a maximum directly following exposure, so the preferred time range for sampling may be clearer than with urine. However, blood concentrations of toxicants may vary with the exposure route; ingested toxicants usually require more time to reach the blood stream than inhaled or dermally absorbed doses. Furthermore, blood measurements are more likely than urine measurements to reflect the dose available for the target site [3] since the measured dose has not yet been eliminated from the body.

The major disadvantages of blood measurements are the venipuncture required to obtain the sample and the low toxicant concentrations. Unfortunately, the invasive nature of venipuncture sampling limits researchers' ability to obtain samples from children or to get high participation rates in large-scale studies. In addition, when samples can be obtained, the amount of blood available to perform the analysis is often limited; therefore, ultrasensitive analytical techniques may be required. Analysis of blood is further complicated by the inherently low toxicant concentrations that are generally present in blood (pg/g or parts per trillion) when compared with urinary metabolite concentrations (mg/l or parts per billion).

For most researchers, the disadvantages of blood measurements have far outweighed the advantages. In fact, most of the scientific literature detailing biological monitoring of contemporary pesticides describes urinary assays [2]. However, several methods involving blood, serum, or plasma measurements of a variety of contemporary pesticides have been published [4–29]. The pesticides measured using these methods include primarily organophosphate and carbamate insecticides. The vast majority of these methods were developed for forensic applications or for diagnosis of acute pesticide intoxication and have limits of detection in the parts-per-billion to the parts-per-million range. In all cases, these methods lack the sensitivity and/or the selectivity to measure pesticides in blood or blood products resulting from incidental exposures.

We have developed a sensitive and accurate method for quantifying 29 contemporary pesticides in human serum or plasma. Our method employs a simple solid-phase extraction followed by a highly selective analysis using isotope dilution gas chromatography–high-resolution mass spectrometry (GC–HR-MS). We have used this method to measure concentrations of these pesticides in the plasma of women living in New York City.

2. Experimental

2.1. *Materials*

All native pesticides and metabolites, except propoxur, bendiocarb, 1-naphthol, parathion, and tetrahydrophthalimide, were obtained from Chem Service (West Chester, PA, USA). Propoxur and bendiocarb were obtained from the EPA repository (Research Triangle Park, NC, USA). Parathion and tetrahydrophthalimide were purchased from AccuStandard (New Haven, CT, USA) and Aldrich (Milwaukee, WI, USA), respectively. All isotopically labeled standards were synthesized by Cambridge Isotope Laboratories (Andover, MA, USA) with the exception of trifluralin, chlorthal-dimethyl, and malathion which were purchased from C/D/N Isotopes (Quebec, Canada) and 1-naphthol which was synthesized in-house. Ammonium sulfate and anhydrous sodium sulfate were purchased from EM Industries (Gibbstown, NJ, USA) and Mallinckrodt (Paris, KY, USA), respectively. OASIS and C_{18} solid-phase extraction columns were purchased from Waters Corporation (Milford, MA, USA) and Varian Analytical Supplies (Walnut Creek, CA, USA), respectively. All solvents were analytical grade and were purchased from Burdick and Jackson (Muskegon, MI, USA). All chemicals and solvents were used without further purification. All reagents were made daily with bioanalytical grade I water, which was prepared in-house using a Solutions 2000 water treatment system (Solutions Consultants, Jasper, GA, USA).

2.2. *Native standards*

Individual stock solutions were prepared by dissolving 3-mg amounts of each standard into 15 ml toluene and mixing well. The stock standards were divided into aliquots, flame sealed in ampules and stored at 2208C until used.

2.3. *Internal standard*

Stock internal standard solutions were prepared by dissolving 5 mg of each stable isotope labeled standard into 50 ml toluene and mixing well. Exceptions to this stock preparation were carbofuran, alachlor, metolachlor, and chlorpyrifos which were purchased as 100 mg/ml solutions in methanol or nonane. An internal standard spiking solution was prepared by diluting the same amount of each stock solution (including the ones purchased in methanol and nonane) with acetonitrile to a concentration of 10 pg/ml. These standards were divided into smaller aliquots, flame sealed in ampules and stored at 2208C until used.

2.4. *Calibration standards*

From the stock native standards, 10 working standard sets (0.25, 0.5, 2, 5, 10, 20, 50, 100, 200 and 400 pg/ml) were created to encompass the entire linear range of the method. The native standard concentration in each standard set was varied as described above, but the labeled internal standard concentrations were kept constant at 100 pg/ml. The standard sets were divided into aliquots, flamesealed in ampules and stored at 2208C until used.

2.5. *Quality control materials*

Quality control (QC) materials were prepared from residual sera from multiple donors from Cincinnati, Ohio, purchased from the Red Cross. Sera were combined and well mixed. Particles larger than 0.2 mm were filtered from the pooled serum using a sterile filtration apparatus. The filtered serum was split into three pools of equal volume. One pool was not enriched, and therefore reflected the native or endogenous concentrations of each pesticide in the serum. The other two pools were enriched with the pesticides at two different levels. Thus, QC pools with native, low (¯15 pg/g), and high (¯50 pg/g) pesticide concentrations were obtained. Following enrichment, all pools were mixed for 24 h under refrigeration. Serum from each pool was dispensed in 4-ml aliquots into vials. The vials were capped, labeled, and stored at 2208C until used. The mean concentration and the analytic variance by the repeat measurement of at least 20 samples in different analytical runs were determined for each QC pool. A QC run was considered unacceptable if one of the following events occurred: (1) the QC sample result for the current run was outside either the upper or the lower 99% control limit; or (2) the QC sample results for the current and most recent previous run were both outside the same upper or lower 95% control limit. In instances where a QC run was considered out-of-control, data generated in the run were considered invalid and the entire run was repeated.

2.6. *Laboratory reagent blanks*

Because virtually all serum samples tested had detectable levels of at least one of the pesticides or metabolites of interest, laboratory reagent blanks consisted of 4 ml of freshly prepared water. The blank contained the same water used in the daily preparation of reagents. The laboratory reagent blanks were prepared in the same manner as unknown samples and were used to ensure that contamination did not occur at any step in the preparation process.

Concentrations of the pesticides in the blank samples were required to be less than the limits of detection (LOD) for the run to be considered acceptable. Data generated in runs with concentrations in the blank sample above the LOD were considered invalid and the analysis was repeated.

2.7. *Sample preparation*

Unknown serum or plasma samples, QC materials, and laboratory reagent blanks were prepared identically. All sera, reagents, and standards were brought to room temperature. A 4-g aliquot of serum/plasma was weighed into a test tube. The serum/plasma was spiked with 100 ml of the working internal standard, mixed, and allowed to equilibrate for approximately 5 min. The serum proteins were denatured with 4 ml of saturated ammonium sulfate. The denatured serum was centrifuged at 3400 rpm for 5 min. An OASIS SPE column was preconditioned with 2 ml methanol followed by 2 ml water. The supernatant was passed through the column and discarded. The column was dried using 20 p.s.i. vacuum for 20 min. The SPE column was eluted with 4 ml methylene chloride. The eluate was passed through a cartridge which contained approximately 1 g anhydrous sodium sulfate and was collected. The extract was concentrated to about 500 ml using a TurboVap evaporator (Zymark, Hopkinton, MA, USA) set at 378C and 15 p.s.i. head pressure of nitrogen. The concentrate was transferred to a 1-ml conical vial. A 10-ml aliquot toluene was added to the vial as a keeper solution and the sample was allowed to evaporate to approximately 10 ml at ambient temperature. The vial was capped and stored under refrigeration until analyzed.

2.8. *Instrumental analysis*

Two microliters of the concentrated extract were analyzed using splitless injection gas chromatography–high-resolution mass spectrometry (GC–HR-MS). The analyses were performed using a Hewlett-

Packard 6890 1 gas chromatograph (GC; Wilmington, DE, USA) interfaced to a MAT 900 trap mass spectrometer (MS) (ThermoFinnigan, Bremen, Germany) equipped with a CTC A200S autosampler and operated using ICIS v8.3 software. Separation was achieved on a 30-m J&W (Folsom, CA, USA) DB-1701MS ([14% cyanopropylphenyl]-methyl polysiloxane, 0.25 mm film thickness, 0.25 mm I.D.) capillary column. Helium was used as the carrier gas with a linear velocity of 35 cm/s. The injector and transfer line temperatures were 240 and 2708C, respectively. The initial column temperature, 1008C, was held for 1 min, increased to 1808C at 158C/min, held for 2 min, increased to 2218C at 38C/min, then finally increased to 2808C at 258C/min and held for 5 min. The MS was operated in single ion monitoring (SIM) mode. The initial accelerating voltage was 5000 and the resolution was 10 000 as definedat 10% valley. Perfluorokerosene(PFK) ions were used as lock and calibration masses.

One ion each was monitored for the pesticide and its respective isotopically labeled internal standard. In the few cases where no labeled standard was available for a particular pesticide, the nearest labeled standard in the same retention time window was used. The monoisotopic masses for each ion monitored for the pesticides and their respective internal standards, the ion types (i.e., fragment or molecular ion), ion composition, retention windows for analysis, and relative retention times are shown in Table . The appropriate analysis specifications were recorded in an acquisition program initiated immediately after autoinjection of the sample into the GC. The total analysis time per sample was about 30 min.

2.9. *Data processing and analysis*

Data were processed using ICIS Quan software (version 8.3, ThermoFinnigan) which was supplied with the mass spectrometer. In Quan, the detection and baseline thresholds were set at 40 and 4, respectively, and the minimum peak width was 1. In addition, the background signal was subtracted and all data were smoothed (three point smooth). The retention times and areas were electronically downloaded into an R:BASE 4.5 1 1 database (Microrim, Redmond, WA, USA) where the ratios of the native and internal standard ions were automatically calculated.

2.10. *Quantification*

Calibration curves were constructed with 10 different pesticide concentrations plotted against the response factors. Response factors were calculated as the area of the native pesticide ion divided by the area of the isotopically labeled pesticide ion. At least five repeat determinations were performed for each concentration on the calibration curve.

Calibration standard concentrations encompassed the entire linear range of the analysis. The lowest standard concentrations were at or below the LOD to ensure linearity and accuracy at the low concentration end. Linear regression analyses of the calibration plots provided slopes and intercepts from which unknown sample concentrations could be determined.

2.11. *Method validation*

The analytical LOD for the method was calculated as $3s_0$, where s_0 was estimated as the y-intercept of a linear regression analysis of a plot of the absolute standard deviation versus the concentration [30].

Twenty serum samples whose endogenous pesticide concentrations were well-characterized were used to evaluate recoveries. Prior to extraction, four samples were spiked with pesticides to a final concentration of 32 pg/g and four were not spiked. The samples were extracted as previously described. Control samples were extracts of the unspiked serum spiked after extraction with the pesticides to final concentrations of 32 pg/g. The extracts of all samples were spiked with the internal standard to correct for instrumental variation during analysis. The recoveries were determined as the ratios of spiked samples to the control samples.

The method accuracy was determined by enriching serum samples with a known amount of the pesticides, preparing and analyzing the samples, and then comparing the calculated and the expected concentrations. Linear regression analyses were performed on plots of the calculated concentrations

Table 1
High-resolution mass spectral analysis specifications

Analyte	Ion type	Monoisotopic mass	Ion composition	Retention window	Relative retention time
2-Isopropoxyphenol (IPP)	M	152.0837	$C_9H_{12}O_2$	1	1.00
Ring-$^{13}C_6$-2-IPPl	M 1 $^{13}C_6$	158.1039	$^{13}C_6{}^{12}C_3H_{12}O_2$	1	1.00
Dichlorvos (DCV)	F	184.9771	$C_4H_7ClO_4P$	1	1.20
Dimethyl-D_6-DCV	F 1 D_6	191.0147	$C_4D_6HClO_4P$	1	1.19
Carbofuranphenol (CFP)	M	164.0837	$C_{10}H_{12}O_2$	1	1.26
Ring-$^{13}C_6$-CFP	M 1 $^{13}C_6$	170.1039	$^{13}C_6{}^{12}C_4H_{12}O_2$	1	1.26
Phthalimide (PI)	M	147.0320	$C_8H_5NO_2$	1	1.90
Ring/carboxyl- $^{13}C_4$-PI	M 1 $^{13}C_4$	151.0454	$^{13}C_4{}^{12}C_4H_5NO_2$	1	1.90
Tetrahydrophthalimide (THPI)	M	151.0633	$C_8H_9NO_2$	1	2.01
Ring-D_6-THPI	M 1 D_6	157.1010	$C_8D_6H_3NO_2$	1	1.99
DEET	M	190.1232	$C_{12}H_{16}NO$	1	2.03
Dimethyl-D_6-DEET	M 1 D_6	196.1608	$C_{12}D_6H_{10}NO$	1	2.02
1-Naphthol (1N)	M	144.0575	$C_{10}H_7OH$	1	2.10
Ring-$^{13}C_6$-1N	M 1 $^{13}C_6$	150.0776	$^{13}C_6{}^{12}C_4H_7OH$	1	2.10
PFK[a]	L	130.9920	n/a	1	n/a
PFK[a]	C	180.9888	n/a	1	n/a
Trifluralin (TFL)	F	264.0232	$C_8H_5N_3O_4F_3$	2	2.27
Dipropyl-D_9-TFL	F 1 D_3	267.0420	$C_8D_3H_2N_3O_4F_3$	2	2.24
Propoxur (PPX)	F	152.0837	$C_9H_{12}O_2$	2	2.30
Phorate (PHT)	M	260.0128	$C_7H_{17}O_2PS_3$	2	2.32
Diethoxy-$^{13}C_4$-PHT	M 1 $^{13}C_4$	264.0262	$^{13}C_4{}^{12}C_3H_{17}O_2PS_3$	2	2.32
Bendiocarb (BCB)	F	166.0630	$C_9H_{10}O_3$	2	2.47
PFK[a]	L	168.9888	n/a	2	n/a
PFK[a]	C	268.9824	n/a	2	n/a
Terbufos (TBF)	M	288.0441	$C_9H_{21}O_2PS_3$	3	2.61
Diethoxy-$^{13}C_4$-TBF	M 1 $^{13}C_4$	292.0576	$^{13}C_4{}^{12}C_5H_{21}O_2PS_3$	3	2.61
Diazinon (DZN)	M	304.1011	$C_{12}H_{21}N_2O_3PS$	3	2.65
Diethyl-D_{10}-DZN	M 1 D_{10}	314.1638	$C_{12}D_{10}H_{11}N_2O_3PS$	3	2.62
Fonophos (FFS)	M	246.0302	$C_{10}H_{15}OPS_2$	3	2.71
Ring-$^{13}C_6$-FFS	M 1 $^{13}C_6$	252.0503	$^{13}C_6{}^{12}C_4H_{15}OPS_2$	3	2.71
PFK[a]	L	230.9856	n/a	3	n/a
PFK[a]	C	292.9824	n/a	3	n/a
Carbofuran (CF)	F	164.0837	$C_{10}H_{12}O_2$	4	2.84
Ring-$^{13}C_6$-CF	F 1 $^{13}C_6$	170.1039	$^{13}C_6{}^{12}C_4H_{12}O_2$	4	2.84
Atrazine (ATZ)	F	200.0703	$C_7H_{11}ClN_5$	4	2.84
Ethylamine-D_5-ATZ	F 1 D_5	205.1017	$C_7D_5H_6ClN_5$	4	2.83
Dicloran (DCN)	M 1 2	207.9620	$C_6H_4N_2O_2^{35}Cl^{37}Cl$	4	2.89
Ring-$^{13}C_6$-DCN	M 1 $^{13}C_6$ 1 2	213.9822	$^{13}C_6H_4N_2O_2^{35}Cl^{37}Cl$	4	2.89
Acetochlor (ACC)	F	223.0764	$C_{12}H_{14}NO_2Cl$	4	3.30
Ring-$^{13}C_6$-ACC	F 1 $^{13}C_6$	229.0965	$^{13}C_6{}^{12}C_6H_{14}NO_2Cl$	4	3.30
Alachlor (ALC)	F	188.1075	$C_{12}H_{14}NO$	4	3.41
Ring-$^{13}C_6$-ALC	F 1 $^{13}C_6$	194.1227	$^{13}C_6{}^{12}C_6H_{14}NO$	4	3.41
Chlorothalonil (CTNL)	M 1 2	265.8786	$C_8^{35}Cl^{37}Cl\ N_2$	4	3.46
PFK[a]	L	168.9888	n/a	4	n/a
PFK[a]	C	230.9856	n/a	4	n/a
Metalaxyl (MXL)	F	206.1181	$C_{12}H_{16}O_2N$	5	3.59
Propionyl-D_4-MXL	F 1 D_4	210.1432	$C_{12}D_4H_{12}O_2N$	5	3.58

Table 1. Continued

Analyte	Ion type	Monoisotopic mass	Ion composition	Retention window	Relative retention time
Chlorpyrifos (CPF)	F	313.9574	$C_9H_{11}Cl_2NO_3PS$	5	3.62
Diethyl-D_{10}-CPF	F 1 D_{10}	324.0202	$C_9D_{10}HCl_2NO_3PS$	5	3.58
Methyl parathion (MP)	M	263.0017	$C_8H_{10}NO_5PS$	5	3.66
Chlorthal-dimethyl (DCL)	F 1 2	300.8807	$C_9H_30_3{}^{35}Cl_3{}^{37}Cl$	5	3.72
Dimethyl-D_6-DCL	F 1 D_3 1 2	303.8995	$C_9D_30_3{}^{35}Cl_3{}^{37}Cl$	5	3.70
Metolachlor (MTCL)	F	238.0999	$C_{13}H_{17}ClNO$	5	3.77
Ring-$^{13}C_6$-MTCL	F 1 $^{13}C_6$	244.1200	$^{13}C_6{}^{12}C_7H_{17}ClNO$	5	3.77
Malathion (MLTN)	F	255.9993	$C_7H_{13}O_4PS_2$	5	3.85
D_{10}-MLTN	F 1 D_5	261.0307	$C_7D_5H_8O_4PS_2$	5	3.81
Parathion (PTN)	M	291.0330	$C_{10}H_{14}NO_5PS$	5	4.08
Diethyl-D_{10}-PTN	M 1 D_{10}	301.0958	$C_{10}D_{10}H_4NO_5PS$	5	4.04
PFK[a]	L	230.9856	n/a	5	n/a
PFK[a]	C	292.9824	n/a	5	n/a
cis-Permethrin (CPM)	F	183.0810	$C_{13}H_{11}O$	6	5.63
Phenoxy-$^{13}C_6$-CPM	F 1 $^{13}C_6$	189.1011	$^{13}C_6{}^{12}C_7H_{11}O$	6	5.63
trans-Permethrin (TPM)	F	183.0810	$C_{13}H_{11}O$	6	5.70
Phenoxy-$^{13}C_6$-TPM	F 1 $^{13}C_6$	189.1011	$^{13}C_6{}^{12}C_7H_{11}O$	6	5.70
PFK[a]	L	180.9888	n/a	6	n/a
PFK[a]	C	192.9888	n/a	6	n/a

M, molecular ion; F, fragment ion; L, lock mass; and C, calibration mass.
[a] PFK, perfluorokerosine.

versus the expected concentrations. With this analysis, a slope of 1.0 would be indicative of 100% accuracy.

2.12. *Human studies*

This method was used to determine the concentrations of pesticides in the plasma of 70 females living in New York City. Ten ml venous blood were collected into Vacutainer tubes containing heparin as the anticoagulant. The tubes were centrifuged at 2500 rpm for 10 min and the plasma was removed to a Qorpak vial. The plasma samples were kept at 2708C until analysis. All protocols were reviewed and approved by a human subjects review committee and complied with all national and institutional guidelines for the protection of human subjects.

3. Results and discussion

The intention of our method was to accurately quantify as many pesticides as possible in a single human serum or plasma sample. The pesticides and metabolites selected for this method represent a variety of pesticide classes and applications. The neurotoxic carbamate and organophosphate pesticides were chosen due to their relatively high use in many agricultural and residential settings. Other high-use pesticides or repellants such as atrazine, alachlor, DEET, and permethrin were measured as well. Certain fungicides and/or their metabolites were added to provide a well-rounded complement of pesticides. In addition, all pesticides selected were required to be amenable to separation by GC. The analyte measured, parent pesticide, and pesticide class are listed in Table 2.

Obtaining adequate, although in many cases not optimal, recovery for analytes was a tedious and challenging process due to the diverse structural characteristics of the pesticides and metabolites chosen for analysis in this method. We evaluated several solid-phase extraction methods for analyte recoveries. We were able to improve the recoveries of some analytes with alternative sorbents; however, we sacrificedthe recoveries of other analytes in those extractions. We selected two SPE methods (C_{18} and OASIS) for further evaluation that provided adequate recoveries to detect the analytes of interest by HRMS. The recoveries of our analytes using C_{18} and

Table 2
Pesticides, metabolites, and their classes

Analyte	Parent pesticide	Class	Use
Acetochlor	Acetochlor	Chloroacetanilide	Herbicide
Alachlor	Alachlor	Chloroacetanilide	Herbicide
Atrazine	Atrazine	Triazine	Herbicide
Bendiocarb	Bendiocarb	Carbamate	Insecticide
Carbofuran	Carbofuran	Carbamate	Insecticide, nematocide
Carbofuranphenol	Carbofuran, carbosulfan	Carbamate	Insecticide, nematocide
Chlorothalonil	Chlorothalonil	Miscellaneous	Fungicide
Chlorpyrifos	Chlorpyrifos	Organophosphate	Insecticide
Chlorthal-dimethyl	Chlorthal-dimethyl	Chloroterephthalate	Herbicide
Diazinon	Diazinon	Organophosphate	Insecticide, acaricide
Dichlorvos	Dichlorvos	Organophosphate	Insecticide, acaricide
Dicloran	Dicloran	Chloronitroaniline	Fungicide
Diethyltoluamide (DEET)	Diethyltoluamide (DEET)	Toluamide	Repellant
Fonophos	Fonophos	Organophosphate	Insecticide
2-Isopropoxyphenol	Propoxur	Carbamate	Insecticide
Malathion	Malathion	Organophosphate	Insecticide,
Metalaxyl	Metalaxyl	Phenylamide	fungicide Acaricide
Methyl parathion	Methyl parathion	Organophosphate	Insecticide, acaricide
Metolachlor	Metolachlor	Cloroacetanilide	Herbicide
1-Naphthol	Carbaryl, naphthalene	Carbamate, polycyclic aromatic hydrocarbon	Insecticide, plant growth regulator
Parathion	Parathion	Organophosphate	Insecticide, acaricide
cis-Permethrin	*cis*-Permethrin	Synthetic pyrethroid	Insecticide
trans-Permethrin	*trans*-Permethrin	Synthetic pyrethroid	Insecticide
Phorate	Phorate	Organophosphate	Insecticide, acaricide, nematocide
Phthalimide	Folpet	*N*-Trihalomethylthio	Fungicide
Propoxur	Propoxur	Carbamate	Insecticide
Terbufos	Terbufos	Organophosphate	Insecticide, nematocide
Tetrahydrophthalimide	Captan, captafol	*N*-Trihalomethylthio	Fungicide
Trifluralin	Trifluralin	Dinitroaniline	Herbicide

OASIS SPE cartridges are shown in Table 3. Although the recoveries of many analytes were comparable for the two SPE sorbents, we chose the OASIS column with a mixed polarity phase because it more efficiently extracted several of the analytes. We surmise the low recoveries of many analytes can be attributed two factors: (1) concentrations of analytes were very low relative to other compounds present in the serum; therefore the column may have overloaded resulting in poor retention of some compounds; (2) elution solvent was too polar to elute nonpolar compounds or too nonpolar to elute really polar compounds. Because many of the pesticides analyzed are highly volatile, we were limited to an elution solvent that had a very low boiling point which made typical elution solvents such as

Table 3
Recoveries of pesticides from serum/plasma using two solid-phase extraction cartridges

Analyte	Percent recovery 6 SD (*n* 5 6)	
	C_{18} [a]	OASIS [b,c]
2-Isopropoxyphenol	41 6 23	48 6 15
Dichlorvos	14 6 10	15 6 10
Carbofuranphenol	63 6 24	80 6 8
Phthalimide	27 6 15	89 6 6
Tetrahydrophthalimide	25 6 14	91 6 8
DEET	40 6 8	43 6 4
1-Naphthol	16 6 12	12 6 10
Trifluralin	12 6 3	15 6 8
Propoxur	48 6 10	61 6 12
Phorate	21 6 13	21 6 11
Bendiocarb	35 6 6	46 6 6
Terbufos	17 6 6	17 6 9
Diazinon	25 6 8	27 6 5
Fonophos	21 6 16	20 6 8
Carbofuran	33 6 15	38 6 10
Atrazine	45 6 15	53 6 12
Dicloran	14 6 18	46 6 23
Acetochlor	22 6 12	23 6 8
Alachlor	23 6 13	21 6 11
Chlorothalonil	11 6 14	14 6 12
Metalaxyl	38 6 12	55 6 9
Chlorpyrifos	18 6 17	21 6 14
Methyl parathion	26 6 20	20 6 16
Chlorthal-dimethyl	15 6 3	18 6 5
Metolachlor	24 6 10	23 6 9
Malathion	15 6 14	22 6 18
Parathion	17 6 17	20 6 18
cis-Permethrin	11 6 7	13 6 5
trans-Permethrin	12 6 4	14 6 5

SD, standard deviation.
[a] 500 mg sorbent bed; 4 g serum.
[b] 200 mg sorbent bed; 4 g serum.
[c] Because the isotope dilution technique was used to automatically correct individual sample recoveries, low and/or variable recoveries did not adversely impact analysis.

methanol, hexane, and toluene less desirable. Although the overall recovery of some analytes was relatively low, this extraction appeared to be the best compromise of all we tested. Frenzel et al. [29] reported much higher recoveries of many of the pesticides we tested from whole blood using kieselguhr columns to perform a pseudo liquid–liquid extraction. Typically with this type of extraction, the recoveries are inversely related to the polarity of the analytes (i.e., recoveries decrease as polarities increase). Because we have previously had little success with this type of extraction column and because our method include many polar metabolites, we did not test these columns with our group of analytes. The low recoveries of some analytes did not adversely affect our analysis because a highly sensitive detection technique was used.

The addition of isotopically labeled standards prior to sample manipulation, a technique known as isotope dilution [31], afforded us many advantages. Chemically, the labeled analogue behaves almost identically to the native pesticide, but they are distinguishable based upon the differences in their masses and/or respective fragment ions. For this reason, the ratio between their ions can internally correct for analyte recoveries in each individual sample. This eliminates the need for recovery surrogates, reduces the error associated with the measurement, and ultimately increases the method sensitivity.

In most instances, the ions chosen for SIM analysis were the most abundant ions whether they were a fragment ion or the molecular ion of the analyte. As is the usual practice when using HRMS, the ratio between the largest and smallest ions in a given retention window should be #1.5, so the accelerating voltage does not scan too low resulting in poor sensitivity or loss of mass lock. In order to better meet these requirements, less abundant ions were used for the analysis of malathion, bendiocarb, propoxur, dicloran, phorate, fonofos, diazinon, and 2-isopropoxyphenol. In addition, the most abundant ion for terbufos required 20 000 resolution to separate it from a PFK ion, so we also used a less abundant ion for monitoring terbufos.

To optimize the sensitivity and selectivity of the analytical method, we obtained chromatographic and/or mass separation of all of the 29 pesticides or metabolites and their respective labeled internal standards. An ion chromatogram reconstructed from individually filtered masses from a 4-pg injection is shown in Fig. 1. Chromatographic resolution was obtained between all but two of the pesticides. Separation was achieved in about 30 min.

Based upon a 4-g sample, the method LODs for each analyte are listed in Table 4. All analytes could be easily detected in the low pg/g range which translates to between 200 and 4 pg detected on-column, depending upon the particular analyte.

A calibration plot for DEET is shown in Fig. 2.

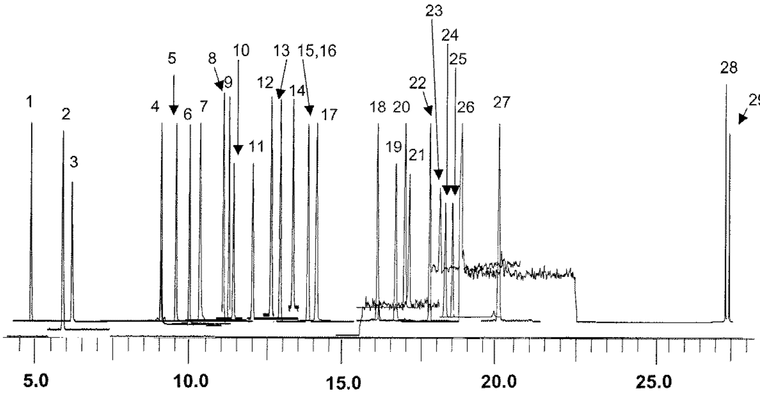

Fig. 1. An ion chromatogram reconstructed from individually filteredions is shown. Four pg of each pesticide were injected on-column. The peaks are identified as follows: (1) 2-isopropoxyphenol, (2) dichlorvos, (3) carbofuranphenol, (4) phthalimide, (5) tetrahydrophthalimide, (6) DEET, (7) 1-naphthol, (8) trifluralin, (9) propoxur, (10) phorate, (11) bendiocarb, (12) terbufos, (13) diazinon, (14) fonophos, (15) carbofuran, (16) atrazine, (17) dicloran, (18) acetochlor, (19) alachlor, (20) chlorothalonil, (21) metalaxyl, (22) chlorpyrifos, (23) methyl parathion, (24) chlorthal-methyl, (25) metolachlor, (26) malathion, (27) parathion, (28) *cis*-permethrin, (29) *trans*-permethrin. All analytes are chromatographically baseline-resolved except carbofuran and atrazine which coelute. Although not shown in this chromatogram, the deuterated internal standards typically elute 5–10 s earlier than the native analyte.

The plots for all analytes were typically linear over three orders of magnitude. Few, if any, matrix effects (e.g., shifting of slope or intercept, higher or lower area counts, etc.) were observed for each analyte; the slopes and intercepts were similar regardless if the analytes were spiked into the matrix or injected as a neat standard. In most cases, the intercepts were statistically indistinguishable from zero ($P > 0.05$). The slopes, intercepts, errors associated with the slope, and correlation coefficients are shown in Table 4.

The method's accuracy was indistinguishable from 100%. Linear regression analyses of plots of the calculated concentrations of spiked samples versus the expected concentrations of the same samples yielded slopes within an acceptable range (0.95–1.05), which is indicative of a high degree of accuracy. These data are shown in Table 4.

A typical quality control Shewart plot is shown in Fig. 3. This plot reflects both intra-day and inter-day variation. The average C.V. for each analyte at concentrations ranging from 10 to 60 pg/g are shown. In most instances, the C.V.s are less than 20%. The imprecision associated with some of the analytes such as dichlorvos and malathion can be partially attributed to the gradual deterioration of these pesticides in the frozen serum pools from which the precision was calculated or from the relative instability of the analyte in the heated injection port of the gas chromatograph.

Overall, the data from the QC materials proved most pesticides were stable in serum over the testing period of approximately 4 months. Those that appeared most unstable in the serum pools were the carbamates and the more reactive organophosphates such as dichlorvos. In fact, we learned from our previous experience measuring carbaryl in serum [32] that carbaryl rapidly decomposed into its metabolite, 1-naphthol, even when samples remained frozen at −70°C. Therefore, when developing this method, we tried to include as many of the carbamate metabolites whose standards were readily available.

Our method is more sensitive and more selective than previously published methods measuring various pesticides in blood or blood products [4–29]. Typical LODs in the literature are three orders of magnitude higher than most of our LODs. However, the imprecision associated with our measurements is typically about double those with higher detection limits.

The specificity of high-resolution mass spectrometry at 10 000 resolution was required to eliminate interfering components in the human serum and

Table 4
Method specifications

Analyte	LOD[a] (pg/g)	Slope[b]	Error of slope[c]	Intercept[b]	R^{2} [b]	Accuracy[d]	C.V.[e]
2-Isopropoxyphenol	3	0.0260	0.6%	2 0.0585	0.999	100	17
Dichlorvos	1	0.0133	0.8%	2 0.0130	0.997	101	13
Carbofuranphenol	1	0.0096	0.8%	0.0026	0.997	100	8
Phthalimide	20	0.0073	0.6%	0.0379	0.998	98	25
Tetrahydrophthalimide	1	0.0059	1.1%	0.0027	0.993	99	14
DEET	10	0.0136	0.6%	2 0.0342	0.999	101	10
1-Naphthol	20	0.0072	0.6%	0.0053	0.999	101	24
Trifluralin	1	0.0226	2.0%	0.0836	0.985	98	27
Propoxur	1	0.0108	1.4%	2 0.0029	0.992	99	19
Phorate	1	0.0023	1.2%	2 0.0004	0.993	99	13
Bendiocarb	5	0.0204	1.7%	2 0.0263	0.986	99	20
Terbufos	1	0.0077	1.0%	0.0049	0.995	97	17
Diazinon	0.5	0.0022	0.4%	0.0008	0.999	101	19
Fonophos	1	0.0136	1.8%	2 0.0609	0.988	103	14
Carbofuran	1	0.0754	1.7%	2 0.3278	0.989	98	30
Atrazine	1	0.0166	0.9%	2 0.0105	0.997	101	17
Dicloran	1	0.0073	1.1%	2 0.0052	0.994	100	13
Acetochlor	1	0.0121	0.7%	2 0.0129	0.998	95	13
Alachlor	1	0.0108	1.1%	2 0.0053	0.994	100	14
Chlorothalonil	5	0.0063	3.9%	2 0.0260	0.954	101	14
Metalaxyl	5	0.0115	0.8%	2 0.0133	0.998	100	25
Chlorpyrifos	1	0.0104	0.5%	2 0.0019	0.999	96	16
Methyl parathion	2	0.0070	3.1%	2 0.0134	0.951	100	20
Chlorthal-dimethyl	1	0.0075	0.4%	2 0.0024	0.999	101	14
Metolachlor	1	0.0063	0.4%	2 0.0034	0.999	101	11
Malathion	12	0.0135	4.5%	2 0.1301	0.906	104	20
Parathion	1	0.0057	1.2%	2 0.0222	0.995	101	17
cis-Permethrin	1	0.0117	0.6%	2 0.0071	0.998	98	31
trans-Permethrin	1	0.0057	0.8%	2 0.0020	0.997	100	28

[a] LOD, limit of detection.
[b] From a linear regression analysis of the concentration versus the area/area internal standard.
[c] Percent error associated with slope of linear regression.
[d] Presented as the slope from a regression analysis of the expected concentration versus the measured concentration.
[e] Calculated from analyses of quality control materials; *n* . 40.

plasma extracts which in turn provided the low detection limits of the method. Analysis at lower resolutions resulted in recurring interferences for many analytes. These specificity requirements precluded the use of single quadrupole or other low resolution mass spectrometers. We did not evaluate this method using tandem mass spectrometry.

In the unspiked pooled serum from Cincinnati that was purchased from the Red Cross, we detected about half of the pesticides and metabolites. These concentrations reflect pesticides that were endogenous in the serum and represent exposure to individuals in the Cincinnati area from whom the serum was collected. These analytes and their mean concentrations are shown in Table 5. Interestingly, similar data were obtained previously from unspiked serum collected from donors in the Chicago area [32]. These data suggest that exposure to pesticides is ubiquitous; the particular classes of pesticides are variable.

We used this method to measure pesticide concentrations in the plasma of 70 women living in New York City. Eight of the pesticides, including organophosphates, carbamates, and fungicides, were detected in greater than 20% of the samples tested. We found chlorpyrifos and dicloran in 96 and 93% of the samples tested, respectively. Phthalimide, tetrahydrophthalimide, and bendiocarb were found in

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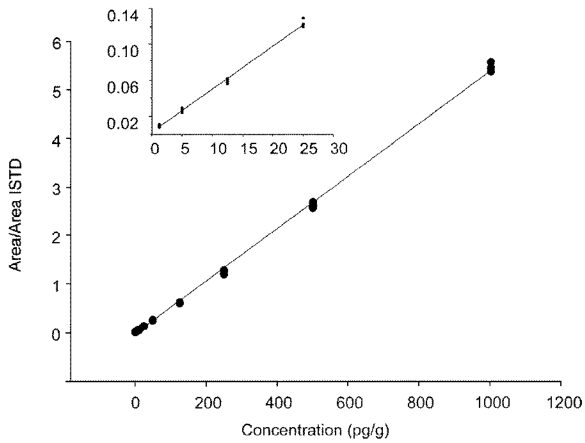

Fig. 2. A calibration curve for DEET over three orders of magnitude is shown. The correlation coefficientis 0.999. The inset shows the low concentration range. These graphs demonstrate the linearity even near the limit of detection. Similar graphs were obtained for all analytes.

Table 5
Mean concentration of selected pesticides in pooled, unspiked serum

Analyte	Mean conc. (pg/g)
Atrazine	2
Chlorpyrifos	9
Chlorothalonil	6
Chlorthal-methyl	4
Dicloran	3
DEET	10
Diazinon	2
2-Isopropoxyphenol	17
Metolachlor	2
Metalaxyl	12
1-Naphthol	46
Phthalimide	28
Tetrahydrophthalimide	4

51, 43, and 56% of the samples, respectively. Diazinon and 2-isopropoxyphenol were seen in 24 and 61% of the samples, respectively. In all instances, the maximum concentrations detected did not exceed 160 pg/g indicating that low LODs are required to measure incidental exposures. Not surprisingly, many of the pesticides such as diazinon, chlorpyrifos, and propoxur, that were detected frequently are widely used in residential applications. Of the pesticides and metabolites measured, nine (alachlor, acetochlor, carbofuranphenol, dichlorvos, DEET, fonofos, parathion, trifluralin, and phorate) were not detected in any of the samples. These data will be published elsewhere in more detail.

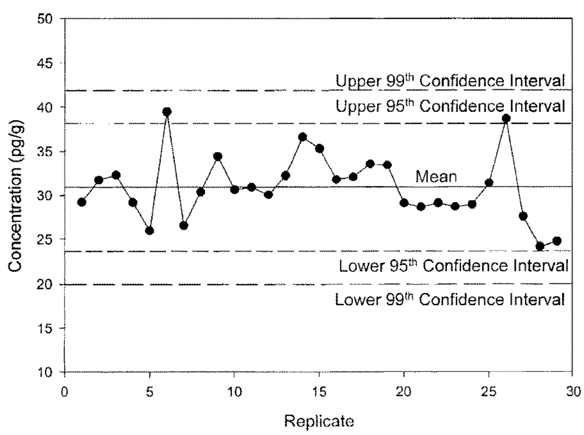

Fig. 3. A Shewart plot of DEET in quality control materials demonstrates the precision of our method. These measurements were made over a period of 4 months. The mean concentration of DEET is 31 pg/g with a total coefficientof variation of 10%.

4. Conclusions

We have developed a highly sensitive method for quantifying serum or plasma concentrations of 29 pesticides and/or their metabolites in humans resulting from incidental, low-level exposures. Our method employs a simple solid-phase extraction with analysis using isotope dilution GC–HR-MS. The LODs are in the low parts-per-trillion range with C.V.sof typically , 20%.

We found detectable concentrations of many widely used residential pesticides in the plasma of urban females. These data confirm the usefulness of our method in detecting incidental exposures to a variety of pesticides. In addition, they confirmthat pesticide exposure is widespread. We plan to further explore pesticide exposures in people living in urban areas. In addition, we will apply this method to measure pesticides in plasma collected from umbilical cords at delivery.

Acknowledgements

We thank Arnetta Downing and Robert Walker for technical assistance. The human studies research was funded by grants to the Columbia Center for Children's Environmental Health from NIEHS (P50 ES0960) and the US EPA (R827027).

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